Effect-directed analysis of aquatic biota

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Summary

This thesis describes a study on the applicability of effect-directed analysis (EDA) to biological matrices, including an expansion of the scope of EDA with the investigation of thyroid hormone disruption and the use of new mass libraries and screening methods for the identification of contaminants.

Chapter 1 provides the background and aim of the studies described in this thesis. It emphasizes the need for broadening the scope of EDA studies by i) investigating to date less studied endocrine disrupting endpoints in EDA, like thyroid hormone disruption, ii) focusing on bioavailable toxicants and thus apply the EDA approach to biological materials and iii) accelerate mass spectral data interpretation and improve identification strategies in EDA that are fundamental parts of a successful EDA study.

To enable the effect-directed identification of bioavailable toxicants in aquatic biota, the development and validation of suitable methods for their extraction from the sample matrix is crucial. Removal of endogenous, natural hormones from the biota extracts is of great importance because their presence in the extracts might lead to the overestimation of the hormone-like activities investigated in the *in vitro* bioassays.

A stepwise-designed sample preparation technique for solid biota, such as tissues and whole body homogenates was developed and validated in Chapter 2. The method consisted of pressurized liquid extraction (PLE) followed by dialysis, gel permeation chromatography (GPC) and normal phase liquid chromatography (NP-LC) as an elaborate cleanup. Besides extracting a broad range of genotoxic and/or hormone-like compounds and enabling the bioassay screening of the extracts, the sample preparation method sufficiently removed the co-extracted natural hormones from the sample extracts by NP-LC fractionation. Furthermore, co-extracted lipids and proteins that often hamper chemical analysis and bioassay testing of biota extracts were also sufficiently eliminated by the applied cleanup steps (dialysis and GPC) and by appropriate dilution of the extracts prior to chemical and bioassay analyses.

Similarly, a sample preparation method that combined solid-phase extraction (SPE) and liquid-liquid extraction (LLE) was developed and validated to be used in EDA of liquid biota (e.g. blood plasma) in Chapter 3. Blood plasma generally contains less lipids than tissue or whole body homogenates. Therefore, a simpler cleanup was sufficient for this type of biological material. The adequate natural hormone removal capability of this method was

proven in addition to its suitability for extracting a broad range of thyroid hormone (TH)-disruptors accumulated in blood plasma.

The extraction and cleanup procedures employed to the solid biota samples (i.e. fish tissues with various fat content) and liquid biota (i.e. cow and polar bear blood plasma) in the method development studies yielded high chemical and biological recoveries, generally above 70% for the spiked compounds. Recoveries of the wide range of spiking compounds from the spiked extracts were chemically determined by gas and liquid chromatography (GC and LC) coupled to mass spectrometry (MS) and recoveries of their toxic activities were biologically determined in the corresponding *in vitro* bioassay.

To demonstrate the applicability of the validated sample preparation technique from Chapter 3 for toxicity profiling and/or further EDA studies, blood plasma samples from polar bear cubs (n=31) were extracted and the capacity of blood-accumulating compounds in the extracts to displace thyroid hormone (TH) from its transport protein, transthyretin (TTR), were measured in the ^{125}I-T_4-TTR-binding assay in Chapter 4. All extracts showed TTR-binding activities. The contribution of target-analyzed halogenated compounds to the total measured TTR-binding activity could be determined based on their plasma concentrations and on their relative TTR-binding potencies. For a few plasma samples, the target compounds only explained half of the observed TTR-binding activity, suggesting the relevance of an EDA study to identify the contaminants responsible for the remaining unexplained part.

Three polar bear plasma extracts were selected for such an in-depth EDA study. This resulted in successful identification of four novel TTR-binding compounds in the plasma extracts as presented in Chapter 5. The identification strategy consisted of compiled mass library screening and cluster analysis of specific halogenated isotope patterns with the aid of instrument related software tools. The identified and analytically confirmed (based on retention time match with the corresponding standards) mono- and dihydroxylated octachlorinated biphenyls (4'-OH-CB201, a monohydroxy octa-CB with unknown substitution pattern and 4,4'-diOH-CB202) and branched nonylphenol were estimated to explain 32±2% of the total measured activity in the polar bear plasma extracts.

In the last study, described in Chapter 6, the developed sample preparation method for solid biota samples, identification strategies and knowledge on toxicity profiling and bioassay-directed identification were all combined. A large set of aquatic abiotic (sediment, suspended particulate matter) and biotic (worms, shrimps, crabs, cockles, and predatory fish) compartments was collected from the Western Scheldt estuary (The Netherlands), the Danish Wadden Sea, and the Scheldt-estuary near Lippenbroek (Belgium). The sample set was extended with passive

sampler silicone rubber sheets collected from the river Meuse near Eijsden and river Rhine near Lobith (The Netherlands), which were considered as "artificial" biota. All samples were extracted and screened for genotoxicity and TTR-binding activity. All biota samples caused high cytotoxicity in the Ames test, which prohibited the quantification of genotoxic activities. All samples showed, however, quantifiable TTR-binding activity. The extracts of passive sampler sheets, worms, crabs and flounders were selected for an in-depth EDA study including fractionation steps to identify the compounds responsible for the measured activities. A number of TTR-binding compounds were tentatively identified in the sample extracts, such as triclosan, nonylphenol, musk ambrette, hydroxylated polychlorinated biphenyls (OH-PCBs), nonsteroidal anti-inflammatory agents (NSAIDs) and perfluorinated alkyl substances (PFASs). Furthermore, the pharmaceuticals citalopram and fluconazole could be identified and confirmed in the selected sample extracts. These compounds had no TTR-binding activity.

Finally, in Chapter 7, the results of this thesis are discussed. The thoroughly validated sample preparation techniques for both solid and liquid biological materials enabled the successful incorporation of bioavailability aspects in the concept of EDA. However, the present study also revealed that EDA studies in general would enormously benefit from high throughput, automated bioassays and fractionation techniques. Also, a clear need for more analytical standards was identified. Novel strategies facilitated the identification of bioactive TTR-binding compounds in various biota samples and emphasized the importance of the continuous extension of mass libraries and compound databases.